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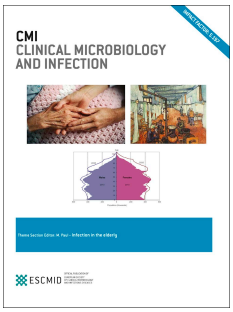

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TORQUETENOVIRUS (TTV): THE HUMAN VIROME FROM BENCH TO BEDSIDE

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ABSTRACT

Torquetenovirus (TTV) is the most abundant component of human virome. Virologists have long ignored this orphan and highly divergent virus, also because TTV cannot be cultured and lacks serology reagents and animal models. Nevertheless, being almost endemic worldwide and insensitive to current antivirals, its monitoring is useful in various conditions. To date, TTV as a marker has proved useful in at least two circumstances: to identify anthropogenic pollution, and assess functional immune competence in immunosuppressed individuals. This review summarizes recent findings about TTV and discusses the main hurdles to translate them into clinical diagnostics.

Keywords: TTV; virome; solid organ transplantation; opportunistic infections; maintenance immunosuppression.

The extensive use of high throughput sequencing on a variety of biological human samples has allowed uncovering many novel viruses, extending tissue distribution of known viral types and, as a result, rapidly revolutionizing many concepts on viral complexity. Thus, it is now evident that many viral agents are present in clinical samples other than the major known pathogenic viruses and that the totality of these agents, defined as human virome, is an integral part of the microbiotic universe that makes us healthy [1, 2]. Although studies are mostly focused on the gut virome, and particularly on its phage component [3], the human blood virome has major implications for immune responses and transfusion safety [4, 5].

Recently, circular, replication initiator protein (Rep) encoding, single stranded DNA (CRESS-DNA) viruses have been discovered in humans. Before the metagenomics era, CRESS-DNA viruses were only known as pathogens infecting plants and animals, but over the past decade they have been found ubiquitously distributed in nature, in a wide variety of invertebrates and vertebrates, including humans [6]. Although human CRESS-DNA viruses are all characterized by small genomes that contain few protein-encoding genes, they are genetically very divergent and belong to distinct viral families [7-10] (Table 1). The effect of these viruses on blood safety and/or human disease is unknown. Recent reports have revealed that CRESS-DNA viruses occupy the largest fraction of the blood virome, and that, among these small DNA viruses, anelloviruses (AVs) account for about 70% of the total virome (Figure 1) [11].

Torquetenovirus (TTV; from *torques* and *tenuis*, Latin for necklace and thin, respectively) is the prototype of AVs [11-14]. The discovery of the first human TTV sequence in 1997 (4) has greatly revolutionized the concept of viral infection, clearly demonstrating the inadequacy of the postulates of Koch in establishing causality in microbial pathogenesis. Its discovery was followed by many other closely related TTV sequences that are now genetically classified in at least 29 major species, each of which consists of numerous strains, that are grouped into the *Alphatorquetenovirus*

genus, Anelloviridae family [16]. This reveals an extremely high degree of genetic heterogeneity, to an extent similar to that found with RNA viruses. In subsequent years, TTV-related, yet clearly distinct viruses, were discovered in humans and animals. These viruses were characterized by smaller genomes than the TTV prototype strains and were therefore called torquetenominivirus (TTMV) [17], and torquetenomidivirus (TTMDV) [18]; of *Betatorquetenovirus* and *Gammatorquetenovirus* genus, respectively (Table 2).

The TTV genome is a circular, negative sense, single-stranded DNA molecule of about 3.8 kb including at least 4 open-reading frames (ORF1 having a hypervariable region) and a GC-rich tract. The sequence divergence is unevenly distributed throughout the genome. The untranslated region (UTR) is well conserved and contains several highly conserved sequences, i.e. showing over 90% identity between isolates. In contrast, the translated region is characterized by a very high degree of diversity [19]. TTV prevalence in population worldwide is extremely high, peaking at > 95% in most cases, and is independent of age, socio-economic standing and health conditions. Multiple TTV species via multiple routes (placental, respiratory, transfusion) are serially acquired very early in life [20]. *In vivo*, the virus appears to replicate mainly in T-lymphocytes [21], but the exact cellular receptor(s) for TTV are still unknown. The only diagnostic approach currently available is detection of the viral DNA in plasma or other clinical specimens. The methods described include several formats of PCR targeted to different regions of the viral genome; however, the one potentially capable of amplifying all of the known genetic forms of TTV is based on a small, highly conserved segment of the UTR. Used in real-time format, this “universal PCR” has led to recognition of the pervasiveness of TTV and revealed that, in individual subjects, viral load may vary between 10^2 and 10^8 DNA copies per ml of plasma [22]. Whether some subjects really are TTV - negative or if this result is merely due to poor sensitivity of PCR or transient absence of replication remains unclear. Most longitudinal studies monitoring TTV viremia by PCR report that

even TTV - negative patients become TTV-positive after immunosuppression [23], suggesting that sanctuaries of viral persistence may exist in body tissues other that peripheral blood. TTV has a remarkable ability to produce chronic infections with no clearly associated clinical manifestations, gaining the status of orphan virus. Such extremely high prevalence and persistence, while being the main deterrent for disease association, may instead be a major requisite for becoming a marker for immunity status. Fast and cheap quantification by real-time PCR and insensitivity to current antivirals also help TTV to be a potential viral indicator. To date, human TTV has proved useful in two main fields: as a marker to detect anthropic pollution and follow up kinetics of functional immune competence.

TTV as a marker of anthropic pollution

The use of bacteria and their associated molecules is commonly used as suitable marker of anthropic pollution of waters, both natural waters or wastewater treated for reuse, and soil [24]. However, bacterial detection suffers from technical problems and significant limitations, that other bioindicators for microbial contamination need to be identified. Additionally, viral contamination of waters and soil can occur regardless of negative bacterial results. Thus, viral markers could provide more accurate health risk assessment data [25, 26]. A number of peculiarities, such as ubiquity and pervasiveness, makes TTV an ideal model for studies of environmental survival and spread. Accordingly, TTV is frequently found in wastewater (38-100%) drinking water (5-12%) [27, 28], and river water [29, 30]. Again, due to its extremely high resistance to known inactivation procedures, TTV testing may be particularly useful after that wastewaters are subjected to procedures of cleaning or treatments with the aim to evaluate the efficiency of these procedures in microbial removal. TTV presence was also revealed in surface (15%) and air (16%) samples collected by hospital settings, thus making wider its possible use as marker of anthropic pollution [31].

TTV as a marker of immune status

Functional immune competence is defined as the ability to mount a protective immune response against an antigenic stimulation. This depends on quality rather than solely amount, e.g. absolute lymphocyte counts, and has sometimes been measured using function surrogates (e.g. immunophenotype). Current assays to measure functional immune competence are either antigen-specific (e.g. ELISpot) or antigen-aspecific (e.g. ImmuKnow™) and/or require availability of target tissue. They are labor-intensive and poorly standardized. These requirements, for instance in solid organ transplantation, cannot be fulfilled because the relevant antigens (other than HLA) are not known, and the target tissue is not available or sufficient, to the point that in most cases immune function is measured with target surrogates, such as the peripheral blood lymphocytes used in cross-matching.

Such extremely high prevalence suggests that TTV has established a fine and successful interaction with the host. To date, investigators do not understand of how effectively immunity controls TTV infection and protects against superinfections by heterologous species of the virus. Kincaid *et al.* showed that TTV encodes a miRNA *in vivo* that targets N-myc (and STAT) interactor (NMI), thus perturbing response to interferons and increasing cellular proliferation in the presence of interferon [32]. These facts support the idea that miRNA mediates immune evasion by antagonizing the host antiviral response and directly contributes to vast ubiquity of these viruses. A precise understanding regarding to what extent immunity modulates TTV replication and the underlying mechanisms is of utmost importance.

Peaks of TTV replication have been observed to occur during sepsis [33], HIV infection [34-36], untreated solid cancer [37], autologous [38] or allogeneic [39] hematopoietic stem cell transplantation, and solid organ transplantation [23, 38-42].

TTV viremia was found to be inversely correlated with the percentage of $CD8^{+}57^{+}$ T-lymphocytes, a cell subset considered a marker of immune competence, in hematological patients affected by lymphoma and myeloma and treated with high-dose chemotherapy supported by autologous hematopoietic stem cell transplantation [38, 43, 44]. Furthermore, the slope of return from peak to baseline TTV values was found to be indicative of the time needed for a patient to recover immune competence before facing the next chemotherapy course [43]. Since hematological patients harbor many confounders (hematologic perturbations due to underlying bone marrow disease or associated chemotherapy), studies have been carried out to replicate these findings in a cleaner model, namely solid organ transplant recipients, across different types of organs, and induction and maintenance immunosuppressive regimens. The main aim of these studies was to identify a TTV viremia threshold discriminating between excessive (too many opportunistic infections and cancers) and inadequate (too many episodes of cellular acute rejection) maintenance immunosuppression. If such threshold can be identified, TTV viremia could be used to gauge and adjust maintenance immunosuppression in solid organ transplant recipients and replace the adjustment procedures based on plasma levels of active ingredient. In fact, it's important to mention that current methods measuring total immunosuppressive drugs in a patient's blood can lead to an overestimation of the amount of functional drug present in blood and are especially used to predict potential toxic effects, not to measure immunosuppressive therapeutic effects.

In children and adult orthotopic liver transplant recipients, TTV viremia correlates with the intensity of maintenance immunosuppression (i.e. calcineurin inhibitors plus azathioprine/mycophenolate mofetil vs. calcineurin inhibitors alone vs. extracorporeal photopheresis, in adult liver transplant recipients) [40, 41]. Patients undergoing solid organ transplantation exhibit marked fluctuations in TTV viremia kinetics and these fluctuations are similar regardless of type of transplanted organ [41]. Since TTV replication occurs mostly in T lymphocytes, it is conceivable that

immunosuppressants are the main determinants of these changes. The extent of lymphocyte depletion early after induction immunosuppression (e.g. ATG vs basiliximab) has indeed an effect on short-term kinetics [45] but it does not affect long-term kinetics of TTV viremia. On the contrary, maintenance immunosuppression is the main determinant of long-term TTV viremia [41]. Interestingly, in some examined patients, the peak of TTV viremia at month 6 post-transplant was preceded by CMV reactivation that occurred in the first 3 months [unpublished data]. This phenomenon suggests that CMV reactivation *per se* induces immune perturbation, or immune conditions that prompted CMV reactivation, also favored TTV replication. Discriminating the order of causation has obvious implications as regards the use of TTV viremia as a prognostic marker.

In parallel to these studies, De Vlaminck *et al.* reported in a milestone paper published in *Cell* that, within a cohort of 199 adult heart and lung transplant recipients, AV loads were associated with the risk of rejection at any time point after transplantation [11]. This study also showed that AVs represent the vast majority of viral sequences in human plasma in the first year after transplantation and that the relative amount directly correlated with both plasma tacrolimus level and oral valganciclovir dose [11]. These results corroborate the initial hypothesis that TTV load is clinically useful (Figure 2). Further evidence in this line of thinking soon followed from other research groups.

In a cohort of 31 adult lung transplant recipients followed up for 2 years, Gorzer *et al.* suggested, using receiver operating characteristic curve analysis, that a threshold level of 9.3 $\log_{10}$ DNA copies of TTV per ml of plasma was predictive for the development of various opportunistic infections in the following timepoint [42]. The same group reported in 2015 in 46 lung transplant recipients that the individual TTV DNA doubling times (range: 1.4-20.1 days) significantly correlated with the pre-transplant TTV levels calculated over 30 or 60 days post-transplantation ($r = 0.61, 0.54$, respectively; both $P < 0.001$), but did not correlate with the tacrolimus mean blood levels. Pre-

transplant TTV levels were not associated with time and level of patient's post-transplant TTV peak load. The authors concluded that the TTV level can be used to gauge immunosuppression only after the patients' initial peak TTV level is reached [23]. Young *et al.* showed that TTV species sequences were 56-fold more abundant in bronchoalveolar lavage from lung transplant recipients at month 5 compared with healthy controls or HIV positive subjects. At that time point, the authors could not find any association with clinical variables, albeit they recognize that longitudinal studies are warranted [46].

Why TTV is not yet part of a diagnostics toolkit ?

Many circumstances have contributed to make TTV a neglected virus of man. The tumultuous pace at which new TTV sequences have been identified and complexity of their interaction with the host have made it difficult to assess a pathogenic potential, if any, of the virus. Difficulties were further enhanced by lack of important investigational tools, including sufficiently sensitive in vitro culture systems and reliable serological assays to demonstrate and investigate viral products and antiviral-specific immune responses. Additionally, TTV have been discovered at the time when HIV and hepatitis viruses dominate a great part of the attention and resources available to medical virologists. Thus, investigation on TTV has so far remained confined to a few laboratories around the world and are a matter of research interest of a small number of scientists. It is therefore inevitable that what we know at this time is only a small fraction of the information that would have needed to fully appraise this uncanny virus. This is particularly true for many aspects of the biology and natural history of TTV. However information to date obtained by a handful of scientists has been sufficient to stimulate scientific curiosity about the virus and the relationships it establishes with the host. Such a curiosity has recently cast light on the substantial role played by TTV in the human virome, and on its considerable impact on the host immune system [22]. Thus, although TTV has not yet been firmly associated with any clinical manifestation, performing the diagnosis of infection could be important. At present, there is no generally standardized diagnostic algorithm

that has been agreed upon or at least discussed among the laboratories that work on TTV. As mentioned above, the PCR methods underwent substantial evolution, and those targeting TTV are several and have different breadths and sensitivities. They can be distinguished in “universal”, which amplify most if not all the human TTVs, and “species-specific” permitting grouping of the virus in one of the 29 species in which TTV have been subdivided. In our laboratory, diagnosis of TTV infection is routinely performed by a quantitative real-time TaqMan PCR assay, designed on a UTR fragment of the viral genome. The diagnosis is focused on the possible pathological consequence of TTV infection in selected populations of patients and is performed for measuring the kinetics of TTV viremia in patients treated with immunosuppressive therapies. It is likely possible that not all TTV species have the same effect and/or impact on the host and, therefore, importance as immune status markers. In 2009, we reported that DNAs of TTV species can differently stimulate Toll-like receptor-9 to release selected pro-inflammatory cytokines [47]. In agreement with this, de Vlaminck *et al.* showed that TTV-8 is by far the most quantitatively prevalent TTV species in human blood [11]. Since current methods use a universal PCR, easier to perform and less expensive than species-specific PCRs, the relative contribution of each genotype to immune modulation will surely be the object of future clinical trials.

Future directions

In addition to its use as potential viral indicator of anthropic pollution [27], TTV may serve as a cheap and easy-to-measure surrogate of functional immune competence, and could prove especially useful in solid organ transplant recipients (the picture is much less clear at the moment for transplant of hematopoietic stem cells). Overall, TTV kinetics are independent of allograft type. While short-term kinetics is mainly driven by type of immunosuppression induction, long-term kinetics is mainly driven by type and plasma level of maintenance immunosuppression.

Which uses can we make of such a marker? TTV viremia could be useful at predicting opportunistic infections [32, 38], although the exact cause-effect relationship remains to be established. TTV viremia has also proven useful at predicting graft rejection in several settings [11]. The latter holds great promise, and TTV viremia could be used to establish tailor-made maintenance immunosuppression.

Other meaningful cohorts remain to be investigated to dissect several aspects of TTV, such as the exact subtype of replication-competent T lymphocyte; lack of suitable animal models forces the design of these investigations to be side studies of clinical trials (e.g. autoimmune disease patients treated with anti-CD3 or anti-CD4 monoclonal antibodies).

Overall, despite that viromics is believed a research branch for big budgets, TTV has shown that small research groups can still make a difference, provided intuition and "relevant" patient populations are in hands.

Acknowledgements

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1 **Table 1.** List of some CRESS-DNA viruses identified in humans

CRESS - DNA viruses	**Anellovirus**	[11]
	Circovirus	[48]
	Cyclovirus	[9]
	Gemycircularvirus	[8]
	Gyrovirus	[49]
	Smacovirus	[10]
	Pecovirus	[10]

2

1 **Table 2.** Taxonomy structure of the Family Anelloviridae

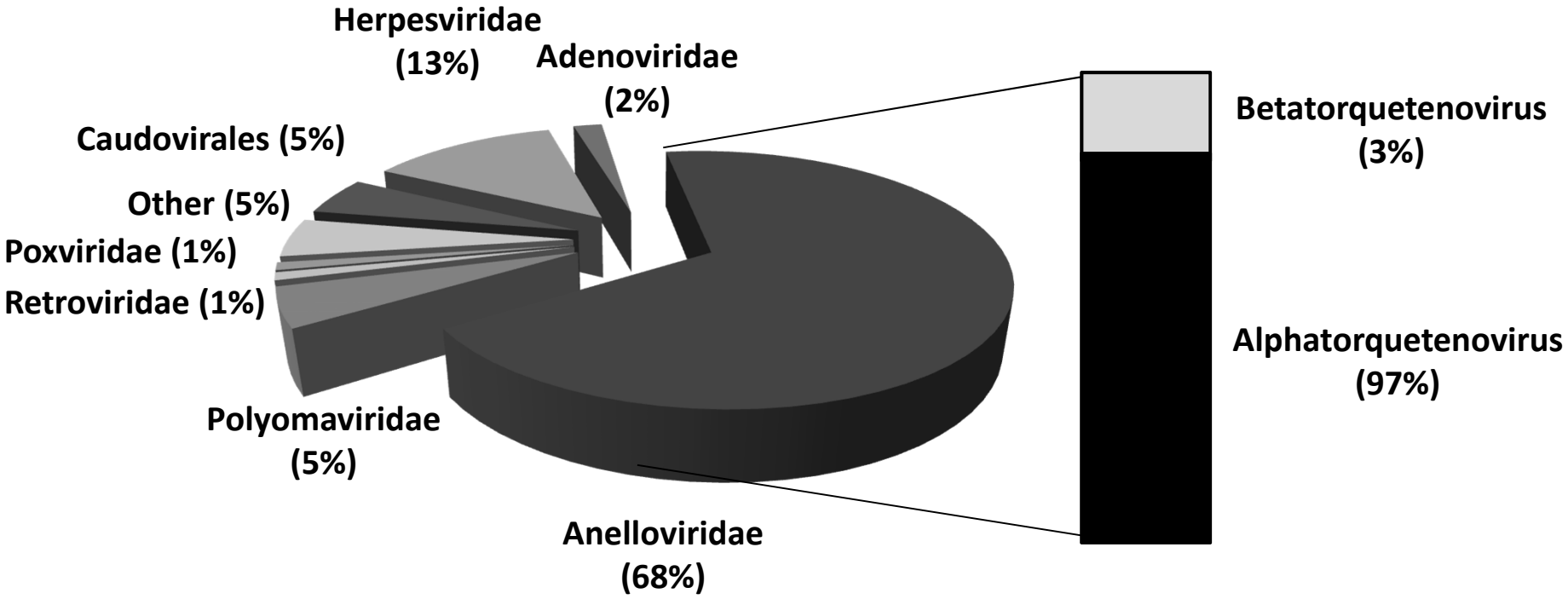
Family	Genus	No. of species	Type species
Anelloviridae	***Alphatorquevirus***	29	Torque teno virus 1
	Betatorquevirus	9	Torque teno mini virus 1
	Gammatorquevirus	2	Torque teno midi virus 1
	Deltatorquevirus	1	Torque teno tupaia virus
	Epsilontorquevirus	1	Torque teno tamarin virus
	Zetatorquevirus	1	Torque teno dourouncouli virus
	Etatorquevirus	1	Torque teno felis virus
	Thetatorquevirus	1	Torque teno canis virus
	Iotatorquevirus	2	Torque teno sus virus 1

2

Legend to Figures

Figure 1. Relative abundance of viruses in plasma virome composition (modified from ref. [11]).

Figure 2. Close interrelationship between immunosuppression and immunocompetence in solid organ transplantation (modified from ref. [11]).


Herpesviridae
(13%)
Adenoviridae
(2%)
Caudovirales (5%)
Other (5%)
Poxviridae (1%)
Retroviridae (1%)
Polyomaviridae
(5%)
Anelloviridae
(68%)
Betatorquetenovirus
(3%)
Alphatorquetenovirus
(97%)